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Evaluation of protease resistance and toxicity of amyloid-like food fibrils from whey, soy, kidney bean, and egg white

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ABSTRACT

The structural properties of amyloid fibrils combined with their highly functional surface chemistry make them an attractive new food ingredient, for example as highly effective gelling agents. However, the toxic role of amyloid fibrils in disease may cause some concern about their food safety because it has not been established unequivocally if consumption of food fibrils poses a health risk to consumers. Here we present a study of amyloid-like fibrils from whey, kidney bean, soy bean, and egg white to partially address this concern. Fibrils showed varied resistance to proteolytic digestion *in vitro* by either Proteinase K, pepsin or pancreatin. The toxicity of mature fibrils was measured *in vitro* and compared to native protein, early-stage-fibrillar protein, and sonicated fibrils in two immortalised human cancer cell lines, Caco-2 and Hec-1a. There was no reduction in the viability of either Caco-2 or Hec-1a cells after treatment with a fibril concentration of up to 0.25 mg/mL.

1. Introduction

Recent reports in the food science literature reveal that amyloid fibrils or amyloid-like fibrils can be formed from various food proteins. These food fibrils are considered a potential new ingredient in food formulations because of their robustness and their rheological behaviour in solutions whilst maintaining low caloric burden (Loveday, Su, Rao, Anema, & Singh, 2011). Amyloid fibrils are good gelling agents/thickeners and can be used as foam stabilisers because of their high length to width ratio (Loveday, Rao, Creamer, & Singh, 2009).

The amyloid fold is believed to be a generic protein conformation that can be assumed by many, if not all, peptides under the appropriate conditions (Chiti & Dobson, 2006). Amyloid fibrils can be formed by native proteins, denatured proteins, protein fragments or peptides. There are over 30 amyloid fibril associated diseases (Chiti & Dobson, 2006), which share a common amyloid aggregate structures and pathological pathways, regardless of the source of protein (Glabe, 2006; Kayed et al., 2003). In the case of Alzheimer's disease, the amyloid β-peptides (Aβ) are generated through proteolysis of the amyloid precursor protein. Amyloidogenic Aβ are 40–42 amino acids long and are the building blocks of amyloid fibrils and plaques (Vassar, 2005).

Soluble fibrillar oligomers are considered by many to be the most pathogenic aggregate species across the disease realm, as assessed by *in vivo*, *in vitro*, and *in silico* studies (Glabe & Kayed, 2006). However, there is some indication that amyloid toxicity is caused by mature fibrils, rather than by fibrillar oligomers (Stefani, 2010; Xue et al., 2009). Studies found a relationship between amyloid toxicity and fibril length, and/or rigidity of β_2-microglobulin *in vitro* (Xue et al., 2009). Bovine insulin has been reported to form either toxic rigid fibrils with parallel β-sheet

Abbreviations: Aβ, amyloid β-peptides; Caco-2, caucasian colon adenocarcinoma-2; FBS, foetal bovine serum; Hec-1a, human endometrial cancer – 1a; INS, insulin; KPI, kidney bean protein isolate; OVA, ovalbumin; PBS, phosphate buffered saline; PDB, protein data bank; PK, Proteinase K; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SPI, soy protein isolate; TEM, transmission electron microscope; ThT, thioflavin T; UniProt, universal protein resource; WPI, whey protein isolate; WST, water soluble tetrazolium.

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conformation or non-toxic filaments with anti-parallel β-sheet character under reducing conditions (Zako, Sakono, Hashimoto, Ihara, & Maeda, 2009).

It has been suggested that the toxicity of fibrillar aggregates is caused by membrane disruption (Glabe & Kayed, 2006). The toxicity may be associated with Ca^{2+} influx and production of reactive oxygen species (Stefani & Dobson, 2003). Membrane disruption could be linked directly to the hydrophobicity and flexibility of the amyloid aggregate (Stefani, 2010). Cell lines with differing membrane architectures may react differently to the same amyloid aggregate (Stefani, 2010).

Oral administration over five days of disease related apolipoprotein A-II amyloid fibrils into mice resulted in fibril deposits along the small intestine already after 2 months of administration and in the tongue, stomach, heart and liver after 3–4 months, where the saline fed control group did not develop symptoms (Xing et al., 2001). Feeding mice with purified amyloid fibrils or with amyloid containing bovine liver was also reported to induce amyloid deposition in a concentration dependent manner (Cui, Kawano, Hoshii, Liu, & Ishihara, 2008). Highlighting the importance of animal age in relation to toxicity of amyloid fibrils, it has been reported that the intestinal uptake of orally administered β-amyloid was elevated in weaning cows compared to post-weaning age cows (Ano et al., 2008). Together, these findings could cause some concern when considering the development of food products containing food-protein born amyloid fibrils.

To date, there has been minimal dedicated research effort towards studying the biological safety of non-disease related amyloid-like fibrils in the context of their potential application in food (Raynes, Carver, Gras, & Gerrard, 2014). There are only a few reports of non-disease related amyloids that are cytotoxic to cell lines *in vitro*. Moreover, the toxic protein fibrils are generally not formed from the native protein but either truncated peptide regions or chemically modified proteins. For example, the amyloid aggregates of the SH3 domain from bovine phosphatidyl-inositol-3′-kinase and the amino-terminal domain of the *Escherichia coli* HypF protein showed high toxicity towards NIH-3T3 cells (Bucciantini et al., 2002). In both cases, fibrillar oligomers rather than the mature amyloid fibrils were reported to be the toxic species. The milk protein κ-casein can form fibrillar structures when reduced and carboxymethylated. Both the mature fibrils and protofibrils have been reported to be toxic towards PC12 cells (Dehle, Ecroyd, Musgrave, & Carver, 2010).

The application of amyloid fibrils in food formulation requires an understanding of physiological processes such as the digestion process of fibrils during passage through stomach and intestine. It has been reported that amyloid fibrils formed from bovine milk derived β-lactoglobulin are readily digested *in vitro* by pepsin (Bateman, Ye, & Singh, 2010) and that the hydrolysates themselves are able to reform new fibrils with different morphologies (Bateman, Ye, & Singh, 2011). These intriguing results stand in some contrast to one of the hallmarks of true amyloid fibrils, that is the high resistance towards hydrolysis by proteases such as trypsin and Proteinase K (Watts et al., 2011).

There clearly is a need to further investigate the *in vitro* digestibility of amyloid and amyloid-like fibrils. The most physiologically relevant proteases (protease-mixtures) in a food context are pepsin and pancreatin. They have served in countless *in vitro* assays as model proteases in simulated environments of the human stomach (pepsin) and small intestine (pancreatin) (Minekus et al., 2014). Proteinase K is a broad-spectrum and highly active protease that has been used extensively to characterise amyloid fibril protease resistance of disease related amyloid fibrils (Watts et al., 2011).

Here, we address the question of whether amyloid fibrils are digestible in the presence of pepsin, pancreatin, and Proteinase K. Four different food protein fibrils (WPI, KPI, SPI, and OVA) were synthesised and studied in detail. The well characterised insulin (INS) amyloid fibrils (Nielsen, Khurana, Coats, & Frokjaer, 2001) were used as a control. Additionally, we address whether fibril toxicity found in disease related amyloids could also be present in non-disease related amyloids aggregates.

Caco-2 cells and Hec-1a cell lines were used for cell viability studies. Two different cell lines were chosen because altered compositions of cellular membranes may influence potential interaction with fibrils (Stefani, 2010). The Caco-2 cell line reproducibly expresses morphological and functional characteristics of the intestinal mucosa, and is one of the most characterized and widely used models of the human intestinal barrier (Ulluwishewa et al., 2014). The Hec-1a cell line is an immortal epithelial cell line derived from endometrial tissue (Kamat et al., 2007). In a parallel study assessing the use of fibrils as nanomaterials, we recently investigated the toxicity of WPI fibrils towards the endothelial cell line Hec-1a and confirmed that WPI fibrils were non-toxic to this cell line (Kaur et al., 2014). To our knowledge, the potential toxicity of WPI, KPI, SPI, and OVA fibrils has not been addressed previously in the context of food.

2. Materials and methods

Whey protein isolate 895 (WPI) was supplied by Fonterra, New Zealand. OVA was purified from day-fresh chicken egg white using DEAE (2-(diethylamino)ethyl) Sephacel anion exchange resin (De Groot & de Jongh, 2003) with stepwise elution of increasing NaCl concentration (20–200 mM), dialysis against distilled water, and 0.22 μm sterile filtration. SPI and KPI were prepared from freshly ground soy bean and kidney bean flour using slightly modified extraction methods (Akkermans et al., 2007; Tang, Zhang, Wen, & Huang, 2010). Briefly, fresh bean flour was stirred in extraction buffer for 45 min (SPI extraction buffer: 30 mM Tris, 10 mM β-mercaptoethanol, pH 8; KPI extraction buffer: 0.5 M NaCl, 25 mM HCl, pH 1.6). The flour suspension was centrifuged and the supernatant was filtered (No.4 filter paper, Whatman), dialysed against distilled water, adjusted to pH 1.6 with concentrated HCl, 0.22 μm sterile filtered, and stored at 4 °C until further use. Bovine insulin was purchased from Sigma–Aldrich (Castle Hill, Australia). Protein concentrations were determined with the Biuret assay.

2.1. Fibril formation

WPI, SPI, KPI fibrils were formed using a 10 mg/mL solution of the respective protein in distilled water, adjusted to pH 1.6 (HCl) and incubated at 80 °C for 22 h, a common treatment that is used to generate amyloid fibrils (Akkermans et al., 2007; Loveday et al., 2010; Tang et al., 2010; Wang et al., 2011). For the formation of OVA fibrils, a 10 mg/mL OVA solution was reduced in 15 mM β-mercaptoethanol at 37 °C for two hours. The reduced sample was adjusted to 100 mM NaCl, pH 1.6, sterile filtered, then incubated at 80 °C for 22 h (Sagis, Veerman, & van der Linden, 2004; Tanaka et al., 2011). 5.8 mg/mL of insulin (Sigma–Aldrich, Castle Hill, Australia) were dissolved in 100 mM NaCl, pH 1.6, and sterile filtered (Nielsen et al., 2001). The solution was incubated at 60 °C for 22 h. After the heating step, all protein samples were cooled on ice for 10 min and stored at room temperature for 7 days to allow fibril maturation. ThT fluorescence and TEM were used to monitor fibrillation. For the formation of pre-fibrillar aggregates the fibril formation protocols were followed as above but samples were used immediately after the 10 min cooling step. The sonication of mature fibrils was carried out in 1.5 mL microcentrifuge tubes that were placed in a sonicating water bath for 15 min.